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(54) Isolation of microbial oils

(57) The extraction of a microbial or single cell oil, for example comprising one or more polyunsaturated fatty acids (PUFAs), directly from microbial cells is disclosed which avoids the need for solvents. After fermentation, the microbial cells are pasteurised, washed and the cell walls lysed or disrupted by a mechanical (e.g. homogenisation), physical (boiling or drying), chemical (solvents) or enzymatic (cell wall degrading enzymes) technique. The oil (containing the PUFA) is then separated from the resulting cell wall debris. This is achieved by centrifugation, which results in an oily phase (top layer) that contains the oil which that can be separated from an aqueous phase (containing the cell wall debris). The oil can then be extracted and if necessary the PUFA can be purified or isolated from the oil.

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Description

[0001] The present invention relates to the extraction (and then isolation) of a microbial (or single cell) oil, preferably comprising one or more polyunsaturated fatty acids (PUFAs), from single cell (or micro-) organisms. The process of the invention involves the disruption or lysis of microbial cell walls, followed by separating the oil from the resulting cell debris. The invention additionally relates to a microbial oil recovered by this process, preferably having a PUFA.

[0002] Polyunsaturated fatty acids, or PUFAs, are found naturally and a wide variety of different PUFAs are produced by different single cell organisms (algae, fungi, etc). They have many uses, for example inclusion into foodstuffs (such as infant formula), nutritional supplements and pharmaceuticals.

[0003] In most microbial PUFA production processes a microorganism is first cultured in a fermenter in a suitable medium. The microbial biomass is then harvested and treated to enable subsequent extraction of a lipid from the biomass with a suitable solvent. The lipid is usually subjected to several refining steps. Care must be taken during the process because degradation can occur if the lipids are subjected to lipolysis or oxidising conditions, for example heating (in the presence of oxygen) and/or due to lipases or lipoxygenases. The art teaches that to avoid oxidation (such as resulting from breaking open the cells and so exposing the contents to oxygen) PUFAs can be extracted from whole intact cells using a solvent (see WO-A-97/36996 and WO-A-97/37032). The use of solvents is a common way of removing lipids from microbial biomass (WO-A-98/50574).

[0004] Although these extraction processes have been used for several years, the solvent needs to be removed and this results in extra cost. In addition, if the lipid is to be used in a foodstuff, it is important that certain solvents, such as hexane, are removed completely, or only remain in very small quantities. If the hexane is removed by evaporation then this may involve heating, and that not only adds to costs but can cause lipid degradation. Furthermore, with increasing environmental considerations, the use of solvents for the extraction of lipids is becoming increasingly expensive and unpopular.

[0005] The present invention therefore seeks to solve or at least mitigate these problems. The Applicant has found that lipids, such as those comprising a PUFA, can be efficiently extracted from microbial cells without the need for solvent(s).

[0006] Therefore, according to a first aspect of the present invention there is provided a process for obtaining an oil (or fat or lipid, the terms are used interchangeably) from microbial cells, the process comprising (a) disrupting (or lysing) the cell walls of the microbial cells to release the oil from the cells. The (microbial or single cell) oil can then be (b) separated from at least part of the resulting cell wall debris. One can then (c) recover, purify or isolate the microbial oil (or one or more PUFAs). A good yield of the oil can be achieved using this process without the need for a solvent. Preferably the oil will comprise one or more PUFAs.

[0007] Recent PUFA preparation processes advocate keeping the microbial cells intact (WO-A-97/36996). This publication describes a PUFA production process where a microbial biomass is generated by fermenting a microorganism, and following fermentation the cells are heated (usually pasteurised). Water is removed from the biomass, and the resulting material extruded to form porous granules. The PUFA is then extracted from the intact cells inside the granules by contact with a solvent, usually hexane. The hexane is then evaporated to produce a crude oil. Throughout this process the cells are kept intact to prevent oxygen in the atmosphere contacting the PUFAs and causing undesirable oxidation. However, it has now been found that a good quality PUFA oil can be achieved if the cells are in fact lysed: any potential oxidation by the atmosphere is more than compensated by the advantage of avoiding the need for solvents.

PUFAs and microorganisms

[0008] In the present invention the microbial cells (or microorganism) may be a bacteria, yeast, algae or fungi. Preferred fungi are of the order *Mucorales.* The fungus may be of the genus *Mortierella, Phycomyces, Entomophthora, Pythium, Thraustochytrium, Blakeslea, Rhizomucor* or *Aspergillus.* Preferred fungi are of the species *Mortierella alpina, Blakeslea trispora* and *Aspergillus terreus.*

[0009] Preferred yeasts of the genus *Pichia* or *Saccharomyces,* for example *Pichia ciferrii.* Bacteria can be of the genus *Propionibacterium.* Suitable algae are dinoflagellate and/or belong to the genus *Crypthecodinium, Porphyridium* or *Nitschia,* for example are of the species *Crypthecodinium cohnii.*

[0010] Preferred PUFAs are C18, C20 or C22 Q3 or Q6 PUFAs. Preferred PUFAs include:

docosahexaenoic acid (DHA);
γ-linolenic acid (GLA);
dihomo-γ-linolenic acid (DGLA);
arachidonic acid (ARA); and
eicosapentaenoic acid (EPA).

[0011] GLA and ARA can both be obtained from fungi, such as *Mortierella, Mucor, Pythium* or *Entomophthora.* DHA can be obtained from algae or fungi, such as the dinoflagellate *Crypthecodinium* or the fungus *Thraustochytrium.* EPA can be produced from algae such as *Porphyridium* or *Nitzschia* or from fungi such as *Mortierella.* Preferred PUFAs are ARA and DHA.

[0012] In the process of the invention the microbial cells (or microorganisms) can first be suitably cultured

or fermented, such as in a fermenter vessel containing a culture medium. The fermentation conditions may be optimised for a high oil and/or PUFA content in the resulting biomass. If desirable, and for example after fermentation is finished, the microorganisms may be killed or pasteurised. This may be to inactivate any undesirable enzymes, for example enzymes that might degrade the oil or reduce the yield of the PUFAs.

[0013] The fermentation broth (biomass and culture medium) may then be removed from the fermenter, and if necessary liquid (usually water) removed therefrom. Any suitable solid liquid separation technique can be used. This (dewatering) may be by centrifugation and/or filtration. The cells may be washed, for example using an aqueous solution (such as water) for example to remove any extracellular water-soluble or water-dispersible compounds.

Cell lysis

[0014] The cell walls of the microbial cells can then be disrupted (or lysed). This can be achieved using one or more enzymatic, physical or mechanical methods or techniques, for example at high shear conditions. Physical techniques include heating and/or drying the cells to a sufficient temperature whereby the cell walls are ruptured. This may comprise boiling.

[0015] Enzymatic methods include lysis by one or more enzymes, e.g. cell wall degrading enzymes. The cell wall degrading enzyme may be a lytic enzyme. Other enzymes include (e.g. alkaline) proteases, cellulases, hemicellulases, chitinases and/or pectinases. Other cell wall degrading substances may be used instead of or in combination with one or more enzymes, e.g. salts, alkali, and/or one or more surfactants or detergents. A combination of physical, mechanical and/or enzymatic methods is also contemplated.

[0016] If a mechanical technique is employed this may comprise homogenisation, for example using a homogeniser. This may be a ball mill or any other machine able to disrupt the cell walls. Suitable homogenizers include high pressure homogenizers (for example at a pressure of 300 to 500kg/cm^2 or bar) such as a polytron homogenizer. Other homogenization techniques may involve mixing with particles, e.g. sand and/or glass beads (e.g. use of a bead mill). Alternative mechanical techniques include the use of milling apparatus, for example homoblenders. Other methods of disrupting the cell walls include ultrasound, spray drying and/or pressing or appliance of high pressures. This last technique is called cold-pressing: it may be performed at pressures of 100-600 bar (kg/cm^2), such as 150-500 bar, optimally from 200-400 bar.

[0017] Homogenization is the preferred method of disrupting the cell walls. There may be from 1 to 3 passes through the homogeniser, either at high and/or low pressures. For example one may use a Gaulin™ homogenizer, at a pressure of from 300 to 900, such as 400 to 800, and optimally 500 to 600 or 700 bar (Atm or kg/m^2). Lower pressures may be employed if required, e.g. from 150 to 300 bar. Hence working pressures can vary from 150 to 900 bar depending on the type of homogeniser, number of passes, etc.

[0018] Although cell lysis can be performed chemically (e.g. using a solvent) this is preferably not employed as the process is desireably solvent-free.

[0019] The disruption of the cell walls may be performed either on the broth resulting from fermentation or on the (optionally washed and/or concentrated) biomass (e.g following solid liquid separation).

[0020] In order to improve cell wall disruption, disruption may be performed at a dry matter content of about 10 to 200g/l. This may be on the fermentation broth, for example after fermentation, or it may be derived from the broth, for example after the broth has been subjected to de-watering and/or solid/liquid separation.

Separation of oil from cell debris

[0021] The microbial oil is then separated from at least part of the cell wall debris formed. At this stage the PUFA may be in an oily or lipid layer. This may be a top or upper layer, which is (or has risen) above an aqueous layer containing cell wall debris. The oily layer comprising the PUFA can then be separated from the aqueous phase. One or more surfactants or detergents may be present or added to assist this process.

[0022] The separation of the oil from at least some of the cell wall debris is preferably achieved or assisted by using a mechanical method, in particular by centrifugation. Suitable centrifuges can be obtained from Westfalia™ (semi- and industrial scale) or Beckman™ (e.g. laboratory centrifuges). Centrifugation (e.g. for a laboratory scale centrifuge) may last for from 2 to 8, such as from 3 to 7, optimally from 4 to 6 minutes (residence time). The centrifugal force (g) may be from 2,000 to 10,000, such as from 3,000 to 8,000, optimally from 4,000 to 6,000g, or from 7,000 to 9,000g, although centrifuges can be employed that have g-forces up to 12,000g or even 15,000g. One or more centrifugations may be necessary. The maximum g force may be lower if using certain centrifuges, for example this may be 6000g if using a Westfalia™ centrifuge (e.g. model NA-7). The flow rate may be from 100-500 litres/hour, such as 150 to 450 1/hr, optimally from 200 to 400 1/hr. Centrifugation may result in either a 2-phase system (a fatty or oily top layer and a lower aqueous layer) or a 3-phase system (a fatty or oily top layer, a middle aqueous layer and a bottom layer, usually containing the cell debris).

[0023] The oil may be free of any carotenoids, e.g. β-carotene. Following disruption and separation the process of the invention may further comprise extracting, purifying or isolating the oil or one more PUFAs.

[0024] One advantage of the process of the invention is that one can avoid the need for any solvents. (In this context solvents exclude water, since the culture medi-

um is usually aqueous and the cells may be washed with water). Thus, no (e.g. organic) solvent may be employed either in the stage of disrupting the cell walls, or in the separation of the PUFA from at least part of the cell wall debris. Preferably, no (e.g. organic) solvent is used either in the extraction, purification or isolation of the oil or one or more PUFAs. Thus, in essence, the process is solvent-free. Thus stages (a), (b) and optionally also (c) can be performed without an (e.g. organic) solvent, for example without the need of a solvent for the oil (or PUFA), e.g. an alkane such as hexane.

[0025] Preferably, the use of a surfactant can also be avoided, and each or both of the disruption and separation stages (a) and (b) can also be performed without the need of a surfactant, for example in the absence of any detergents.

[0026] If the oil comprises a PUFA, then the PUFA is preferably predominantly (such as greater than 50%, 70% or even 90% or 95%) in the form of triglycerides. The oil may be refined and/or treated with an acid and/or alkali if required.

[0027] The PUFA (or oil containing a PUFA) may be subjected to further downstream processing, for example degumming, neutralisation, bleaching, deodorization, or winterization.

Overall protocol

[0028] A preferred process of the present invention therefore comprises:

(a) culturing microbial cells under conditions, for example whereby they produce a microbial oil or at least one PUFA;
(b) heating or pasteurising cells, for example to kill the cells and optionally to inactivate any undesirable enzymes;
(c) optionally removing an (aqueous) liquid (such as dewatering), for example by centrifugation, filtration or other solid liquid separation technique;
(d) optionally, washing the microbial cells, for example with water, preferably to remove extracellular water-soluble or water-dispersible compounds;
(e) disrupting or lysing the cell walls of the microbial cells, for example by a physical, enzymatic or mechanical technique (such as homogenisation, e.g. with an homogeniser or a ball mill). This releases some of the oil and/or PUFA present in the microbial cells. The (mechanical) disruption may be supplemented with or substituted by chemical and/or enzymatic disruption. One can obtain an oil phase and an aqueous phase. The oil phase may contain the PUFA. The aqueous phase may contain cell debris;
(f) separation of the microbial oil (or PUFA) from the cell wall debris, for example separation of the oil phase from the resultant cell wall debris and/or aqueous phase. This may comprise centrifugation, optionally with the addition of one or more salts, a pH shift (towards alkaline), and may involve the presence of one or more cell degrading enzymes, surfactants or emulsifiers;
(g) extraction, purification or isolation of the oil (or of the PUFA from the oil phase), for example resulting in a PUFA-containing oil; and
(h) optionally acid treatment (or degumming), alkali treatment (or neutralisation), bleaching, deodorising, cooling (or winterisation). This may remove undesirable substances such as free fatty acids (FFAs), proteins, phospholipids, trace metals, pigments, carbohydrates, soaps, oxidation products, sulphur, pigment decomposition products, sterols, saturated triglycerides and/or mono- or di-glycerides.

[0029] The heat treatment or pasteurization preferably inactivates or denatures one or more oil (or PUFA) degrading enzymes. The temperature of heating may be from 70 to 90°C, such as about 80°C. It may inactivate or denature enzymes such as lipases and/or lipoxygenases.

[0030] One may add one or more (e.g. water and/or oil-soluble) antioxidants, for example vitamin C, ascorbyl palmitate and/or tocopherol, and this may be done after stage (b), or at a later stage for example after extraction, such as before or after any refining (step (h) above).

[0031] There may be one or more additional heating steps, for example to remove other undesirable compounds or components. For example, heating may take place at an acid pH, for example to remove components such as phospholipids, trace metals, pigments, carbohydrates and/or proteins. Here the temperature may be from 50 to 80°C, such as 55 to 75°C, optimally from 60 to 70°C. The pH may be from 1 to 6, such as 2 to 5, optimally at a pH from 3 to 4. This can result in degumming and/or removal of proteins and/or water-soluble or water-dispersible compounds.

[0032] Alternatively or in addition a further heating step, this time at alkaline pH, may employed. The pH may be from 8 to 13, such as from 9 to 12, optimally at a pH of from 10 to 11. The temperature may be the same as that described in the previous paragraph.

[0033] The invention will now be described, by way of example, with reference to the following Examples which are provided by way of illustration only.

Example 1

Preparation of crude PUFA (ARA) oil from a fermentation broth of *Mortierella alpina.*

[0034] A fermentation broth of *Mortierella alpina* (previously pasteurized at 65°C for 1 hour) containing arachidonic acid (ARA) was homogenized once by means of an MC-4 APV Gaulin™ homogenizer at 600 bar (600 Atm) to disrupt the cell walls. Subsequently the homog-

enized broth was centrifuged by means of a Westfalia™ NA-7 disc centrifuge at maximum speed (about 8,000 rpm, equivalent to about 8,000g at the disc stack) resulting in an arachidonic acid-enriched oily top layer (that was recovered from the centrifuge) and a lower aqueous layer containing the cell debris. A crude PUFA oil was recovered: the yield of oil was 95% (based on the oil in the cell). The crude oil had the following approximate composition: 1 to 2% sterols and cell debris; 3 to 4% phospholipids; 4% monoglycerides; 6% diglycerides; and the remainder being triglycerides.

Example 2: Preparation of crude PUFA (DHA) oil from a fermentation broth of *Crypthecodinium cohnii*

[0035] Following fermentation 20 litres of a fermentation broth (pasteurised at 65°C for 1 hour) of the algae *Crypthecodinium cohnii* was homogenized three times by means of an of APV Gaulin™ homogenizer (type: Lab 60/60-10 TBSX), each time at 600 bar, to lyse the algal cell walls. Subsequently a crude oil was recovered using a labscale centrifuge (Beckman™ JM/6E) by centrifuging the broth in 800ml portions each for 5 minutes at 5000g. This resulted in a DHA-enriched fatty top layer (crude oil) and a lower aqueous layer. A crude PUFA oil was recovered from the fatty top layer.

Claims

1. A process for obtaining an oil from microbial cells, the process comprising:

   (a) disrupting the cell walls of the microbial cells to release the oil; and
   (b) separating the oil from at least part of the cell wall debris formed in (a).

2. A process according to claim 1 wherein:

   (i) the cells are physically, enzymatically or mechanically disrupted, optionally by homogenisation; and/or
   (ii) the oil comprises one or more polyunsaturated fatty acids (PUFAs).

3. A process according to claim 2 which further comprises:

   (c) extracting, purifying or isolating the microbial oil or one or more PUFAs.

4. A process according to any preceding claim wherein the separation in (b) is by centrifugation and/or the separation results in the formation of an oil layer comprising either the microbial oil or the PUFA.

5. A process according to any of claims 2 to 4 wherein the PUFA is a C_{18}, C_{20} or C_{22} Ω-3 or Ω-6 PUFA (optionally ARA, EPA, DHA and/or GLA) and/or is present in an oil or lipid.

6. A process according to any preceding claim wherein the microbial cells are yeast, bacterial, fungal or algal cells.

7. A process according to any of claims 2 to 7 wherein no solvent (such as for the oil or PUFA) is employed in stages (a) and (b), and optionally also in (c).

8. A process according to any preceding claim which comprises, before (a), culturing or fermenting microbial cells under conditions that allow production of the oil, and if necessary pasteurising and/or heating the cells.

9. A process according to any preceding claims wherein the disruption of the cell walls is assisted by one or more cell wall degrading enzymes or surfactants.

10. A microbial or single cell oil comprising a PUFA, or a PUFA, obtained by a process according to any preceding claim.

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EUROPEAN SEARCH REPORT

Application Number
EP 00 30 6601

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	VAZHAPPILLY REMA ET AL: "Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth" JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY,AMERICAN OIL CHEMISTS' SOCIETY. CHAMPAIGN,US, vol. 75, no. 3, March 1998 (1998-03), pages 393-397, XP002152068 ISSN: 0003-021X * page 393, right-hand column - page 394, right-hand column; tables 2,3 *	1-3,5,6, 8,10	C12P7/64 C11B1/00
X	GB 808 128 A (CHALLINOR S W AND DANIELS N W) * page 4, line 100 - line 106 *	1,4,6,8	
X	PREEZ DU J C ET AL: "PRODUCTION OF GAMMA-LINOLENIC ACID BY MUCOR CIRCINELLOIDES AND MUCOR ROUXII WITH ACETIC ACID AS CARBON SUBSTRATE" BIOTECHNOLOGY LETTERS,KEW, SURREY,GB, vol. 17, no. 9, September 1995 (1995-09), pages 933-938, XP000960382 ISSN: 0141-5492 * abstract *	10	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12P C11B

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	23 January 2001	Devijver, K

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 00 30 6601

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 808128 A		NONE	

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82